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Changes in free amino acid levels in sour orange leaves in response to cold stress and during recovery from cold stress

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Abstract

In a previous study, we reported that potted sour orange trees recovering from cold stress attracted more Asian citrus psyllids than the control plants continuously kept under warm condition. In parallel studies, cold-treated plants were shown to have relatively increased amounts of ninhydrin positive material during 5-24 h recovery period from cold stress. Here we report on changes in free amino acid levels in sour orange leaves in the 24 h recovery period following the termination of the chilling treatment. Proline was most abundant amino acid and increased in response to chilling treatment; it remained at higher level than initially during the first 5 h recovery period and then declined to initial level after 24 h. In addition, amino acids, such as serine, glutamic acid, glycine, lysine, isoleucine, tyrosine, arginine and histidine, increased due to cold treatment and/or during recovery after cold treatment, while asparagine, phenylalanine, leucine, alanine and threonine either decreased or remained unchanged in response to cold stress and during immediate recovery period. The special pattern with which levels of different free amino acids change in response to cold stress might have important implications on interactions between Asian citrus psyllids and citrus host plants.

Key words: Amino acids, citrus, leaves, cold stress, sour oranges.

Introduction

Citrus greening disease, also known as Huanglongbing (HLB), is a devastating disease that has caused serious damage to citrus industry in Florida, USA, and other places in the world [1, 2]. The disease causing bacteria (*Candidatus Liberibacter* spp.) is transmitted by Asian citrus psyllid (ACP), *Diaphorina citri* [1, 2]. Since there is no cure for the disease as yet, the problem is managed through controlling the insect vector; primarily by spraying insecticides throughout the year [2-4]. We have, therefore, started studies on interactions between ACP and the host plant [5]. Previously, we reported that plants recovering from cold stress attracted more ACP than the control plants continuously kept under warm condition. In parallel studies, cold-treated plants were shown to have relatively increased amounts of ninhydrin positive material, at least during 5-24 h recovery period from cold stress; changes in various polyphenols and polyamines were are also observed [5].

One reason for increased attractiveness of a host plant for herbivores is increased nutrient levels (e.g., amino acids) providing greater feeding opportunities [6-8]. In fact, outbreaks of herbivore insects have been attributed to changes in amino acid levels in host plant facilitating feeding and fecundity of insect pests [8]. Increases in free amino acid levels have also been observed in leaves of various plant species in response to different stresses [9-12]. It is, therefore, important to extend our previous studies on interactions between ACP and cold-stressed plants by looking at changes in free amino acid levels in sour orange leaves when subjected to the cold stress and during recovery period when greater attractiveness to ACP was observed. Results from this study are described here.

Materials and Methods

The plants: The conditions for growing sour orange (*Citrus aurantium*) plants and cold stress treatment were the same as described in our previous report [5]. Briefly, 8 week old sour orange plants grown from seeds in 10 cm diameter pots filled with a mixture of peat: perlite: vermiculite (1:1:1 by wt) were used for the cold stress experiments. The plants were grown at 28±2°C under a bank of fluorescent lights (128 $\mu m^{-2} s^{-1}$) giving a 14 h photoperiod and were supplied with Peters complete fertilizer every other week. Six replicate plants (8 weeks old) were transferred to a modified refrigerator with fluorescent lights maintained at 6±1°C and a 14 h photoperiod and were kept there for six days before bringing them back to 28±2°C. Leaf samples were taken at the start of cold stress, and at 0 time 5 h and 24 h after the plants returned to warm temperature. In addition, leaf samples from six control plants, continuously kept in warm temperature, were taken at the start of the experiment and also at the end of the experiment; i.e., at the time plants of the other set were placed in cold temperature (6±1°C) and also 24 h after the cold treated plants were brought at warm temperature (28± 2°C).

Amino acid extraction: Cold-stressed and control leaf samples were stored at -80°C until needed for analyses. Before extraction, frozen leaves were pulverized in liquid nitrogen as described previously [13]. A 300 mg aliquot of the frozen leaf powder was transferred to a 60 ml Pyrex test tube to which 25 ml of 80% methanol was added. The mixture was homogenized with a Polytron (model PT 3100) for 30 s (3 times with 10-20 s breaks) at maximum speed. To the homogenized mixture glass bead (5-7) were added and the test tube was placed in a boiling water bath.

The mixture was allowed to boil for 20 min. After cooling, the mixture was filtered through glass wool and the final volume made to 10 ml with 80% methanol.

Purification and derivatization of the extract for amino acid analyses by HPLC: The extract was centrifuged at high speed and passed through 0.45µm filters. A 4 ml aliquot of this extract was loaded on a cation exchange resin (Bio-Rad 50W-X8 [H^+]) which was filled in glass column (100 mm x 9 mm) to a 40 mm height; 1.12 mg of resin was swelled in excess milli-Q water and the resin in the column was equilibrated with milli-Q water prior to extract loading. The column was washed with a three column volume of milli-Q water followed by 6 ml of 6 M NH_4OH to elute bound amino acids from the extract. The eluent was reduced to dryness using SPD 1010 SpeedVac (Thermo Savant, Holbrook, NY USA) and the residue was taken-up in 500 µl of 20 mM HCl and filtered through 0.2µm filter. The amino acids in the filtrate were derivitized using Waters AccQ-Tag following their protocol as described previously [14]. The derivitized amino acids were separated an AccQ Tag (4 µm particle size) (3.9 mm x 150 mm) column fitted in Waters Alliance 2695 HPLC system equipped with a 2475 Multi λ Fluorescence Detector. The chromatographic conditions were maintained exactly as described in the AccQ-Fluor Reagent Kit instruction manual.

Regression curves using standard compounds were developed for each amino acid following the same derivatization and separation techniques described above. Concentrations of individual amino acids in the extracts were calculated using regression equation, and using appropriate dilution factor. The amounts of individual free amino acids are reported as µmoles per mg fresh weight of the tissue.

Data analyses: Each replicate extract was analyzed in triplicate using HPLC chromatographic conditions, statistical analysis on the means of replicate experiments were performed using the ANOVA procedure of the InStat® software, 230 version 3.0 (GraphPad, San Diego, CA) and the Tukey's test of significance between means. Significance was reported if the P-value was at least <0.05.

Table 1. Changes in asparagine, threonine, alanine, leucine, and phenylalanine levels in sour orange leaves as affected by cold stress (6±1°C for 6 days) and during recovery in warm temperatures (28±2°C for 24 h).

	ASP	THR	ALA	LEU	PHE
Initial	8.27±0.84 [a]	4.56±0.34 [a]	7.73±0.26 [a]	3.18±0.06 [a]	4.06±0.31 [a]
T0	5.87±0.26 [b]	4.14±0.18 [ab]	8.24±0.16 [ab]	2.87±0.07 [ad]	1.30±0.03 [b]
T1	2.05±0.07 [c]	4.40±0.08 [ab]	8.34±0.35 [ab]	3.19±0.15 [ad]	3.30±0.02 [a]
T2	3.28±0.21 [c]	3.07±0.18 [c]	6.32±0.56 [ad]	2.12±0.23 [b]	3.41±0.30 [a]

Initial values represented levels of specific amino acid in leaves of plants grown in warm temperature (28±2°C); T0 represents values in leaves of plants sampled immediately after 6 days of cold treatments; T1 and T2 represents values 5 h after and 24 h, respectively, after the plants were taken from cold and kept at warm temperature. Dissimilar letters or numbers represent significant differences (P<0.05) in the values of each amino acid in the control.

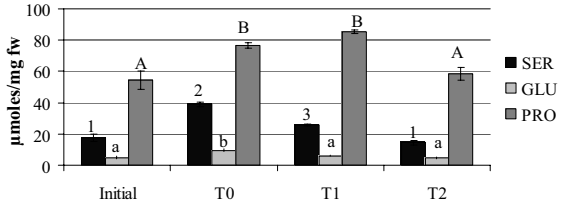

Figure 1. Changes in serine, glutamic acid, and proline levels (high abundance amino acids) in sour orange leaves as affected by cold stress (6±1°C for 6 days) and during recovery in warm temperatures (28±2°C for 24 h). Initial values represented levels of specific amino acid in leaves of control plants grown in warm temperature (28±2°C); T0 represents values in leaves of plants sampled immediately after 6 days of cold treatments; T1 and T2 represents values 5 h after and 24 h, respectively, after the plants were taken from cold and kept at warm temperature. Dissimilar letters or numbers represent significant differences (P<0.05) in the values of each amino acid in the control.

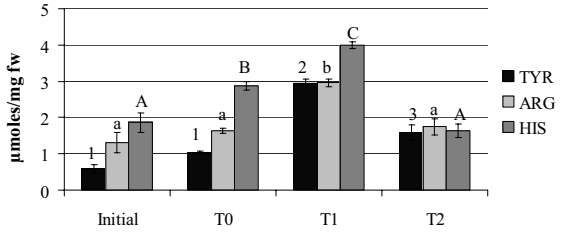

Figure 2. Changes in tyrosine, arginine, and histidine levels (medium abundance amino acids) in sour orange leaves as affected by cold stress (6±1°C for 6 days) and during recovery in warm temperatures (28±2°C for 24 h). Initial values represented levels of specific amino acid in leaves of plants grown in warm temperature (28±2°C); T0 represents values in leaves of plants sampled immediately after 6 days of cold treatments; T1 and T2 represents values 5 h after and 24 h, respectively, after the plants were taken from cold and kept at warm temperature. Dissimilar letters or numbers represent significant differences (P<0.05) in the values of each amino acid in the control.

Results

The free amino acids that increased significantly in response to cold stress were grouped in three categories (i.e., high, medium and low level of abundance) for the purpose of illustration (Figs 1-3), and the amino acids that did not show any significant increases are presented in Table 1. Serine, glutamic acid and proline occurred in higher amounts relative to other free amino acids and are shown in Fig. 1. Cold stress significantly increased the levels of these three amino acids (T0) but 5 h (T1) after returning to warm temperatures only the levels of proline and serine were higher than at T0, and after 24 h (T2) the quantities of all of the three amino acids were reduced to T0 levels (Fig.1).

Changes in the levels of tyrosine, arginine and histidine (of medium level abundance) in response to cold stress and during recovery are shown in Fig. 2. In this case only histidine levels increased at the end of cold period (T0) although levels of all the these three amino acids were significantly higher in leaves sampled 5 h after taking the plant out of cold (T1) into warm temperature (Fig. 2). After 5 h recovery period (T1) the levels of these amino acids declined and after 24 h recovery period (T2) the levels of all the amino acids were significantly lower than in T1 samples but tyrosine and arginine were still higher than the initial values, although histidine quantities in T2 samples returned to initial levels (Fig. 2).

Amounts of glycine, lysine and isoleucine were below detectable levels in the initial sour orange leaf samples (Fig.3). The levels of all these three amino acids significantly increased following chilling (T0) and during 5 h recovery period (T1) while significantly lower amounts of these amino acids were found in samples taken 24 h after cold treatment (T2), and reached below detection limits except isoleucine (Fig. 3). As opposed to serine and glutamine levels that decreased during 5 h recovery period (T1), the levels of glycine, lysine and isoleucine significantly increased in T1 samples compared to T0 samples (Fig. 3). The levels of asparagine, threonine, alanine, leucine and phenylalanine either decreased or remained unchanged in response to cold stress and recovery from cold stress (Table 1).

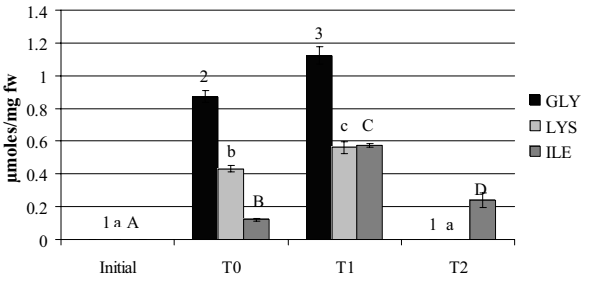

Figure 3. Changes in glycine, lysine and isoleucine levels (low abundance amino acids) in sour orange leaves as affected by cold stress (6±1°C for 6 days) and during recovery in warm temperatures (28±2°C for 24 h).

Initial values represented levels of specific amino acid in leaves of plants grown in warm temperature (28±2°C); T0 represents values in leaves of plants sampled immediately after 6 days of cold treatments; T1 and T2 represents values 5 h after and 24 h, respectively, after the plants were taken from cold and kept at warm temperature. Dissimilar letters or numbers represent significant differences (P<0.05) in the values of each amino acid in the control.

Discussion

The results presented in Figs 1-3 support our earlier suggestion that a number of free amino acids in the sour orange leaves (e.g., serine, proline, tyrosine, arginine, histidine, glycine, lysine, isoleucine) would increase in response to short-term cold stress and during the immediate recovery period [5]. Our hypothesis was based on the results of previous studies [5], where we found that cold-stressed sour orange seedlings (6±1°C for 6 days) that showed increased attractiveness towards ACP also had increased amounts of ninhydrin positive material; along with changes in polyphenols and polyamines [5]. Thus, our results are consistent with reports in the literature that increased levels of amino acids, in response to different stresses, parallel with increased infestation of various insects [6-12]. However, additional studies are now needed to determine whether one or combination of the amino acids that were found to increase in this study are actually preferred food for ACP.

Proline was most abundant amino acid that significantly increased in response to cold stress and during the first 5 h recovery at (28 ± 2°C); these results are similar to previous reports on other citrus cultivars under different experimental conditions[15, 16]. Behmer and Joren [17] reported that in some insects, proline is indeed a preferred feeding material and female preference for proline was greater than males. Thus, in future studies on diet preferences for ACP it would be pertinent to include proline as one of the component in the diet.

In addition to proline, several other amino acids, such as glycine, lysine, isoleucine, tyrosine, arginine, histidine and serine, also increased while asparagine, phenylalanine, leucine, alanine and threonine either decreased or remained unchanged, in response to cold stress and during immediate recovery period. Apparently, these changes might had some contributory effects, by increasing attractiveness and/or by decreasing repulsiveness in the sour orange leaves for the previously observed increased ACP infestation of sour orange leaves cold stress [5]. For example, while lysine is a preferred nutrient for locust (*Locusta migratoria)*, flesh fly (*Sarcophaga bullata*) avoids lysine and prefers glycine [18, 19].

Conclusions

Cold stress and recovery from cold stress in sour orange leaves produce specific changes in levels of amino acids; i.e., while levels of some amino acids increase, levels of several others decrease. It is postulated that some of these changes may play important role in regulating attractiveness of sour orange leaves to ACP by such treatments as observed in previous study.

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